A multiplex PCR assay for simultaneous detection of Corynebacterium diphtheriae and differentiation between non-toxigenic and toxigenic isolates

The importance of identifying Corynebacterium diphtheriae can be appreciated since diphtheria remains endemic in many countries, alongside the risk of epidemic outbreaks and the existence of a large number of non-immunized people worldwide (Galazka & Robertson, 1995; Damasco et al., 2005). The features of infections caused by C. diphtheriae have changed over the decades, and are most clearly emphasized by the emergence of non-toxigenic strains causing atypical diseases, such as endocarditis, septic arthritis or infection in unusual anatomical sites (Mattos-Guaraldi & Formiga, 1998; Efstatiou & George, 1999; Reacher et al., 2000; Mattos-Guaraldi et al., 2003). Rapid and reliable methods are needed for identifying a C. diphtheriae infection, thus aiding in appropriate and timely patient management, and improved monitoring of cases.

The use of multiplex PCR is becoming increasingly important in the diagnosis of infectious diseases, and could be a simple and fast alternative procedure for identification of C. diphtheriae, including for screening for non-toxin-gene- and toxin-gene-bearing strains (Cianciotto & Groman, 1997). In this study, a multiplex PCR assay was developed as a rapid and simple method for the identification of C. diphtheriae, and for the differentiation between non-toxigenic and toxigenic strains among non-pigmented Gram-positive rods.

The study was undertaken with 84 C. diphtheriae strains from the culture collection of the Diphtheria Laboratory, Universidade do Estado do Rio de Janeiro, Brazil, as follows: 74 strains of C. diphtheriae subsp. mitis, 5 of C. diphtheriae subsp. gravis and 5 of C. diphtheriae subsp. belfanti; 33 of the strains were toxigenic and 51 were non-toxigenic; 40 of the strains were non-sucrose fermenting and 44 were sucrose fermenting. These C. diphtheriae strains were isolated from skin infections (n=34), blood-stream infections (n=4) and respiratory tract infections (n=46). This study also included three non-toxigenic Corynebacterium ulcerans, in addition to five non-toxigenic Corynebacterium pseudotuberculosis clinical strains kindly provided by Vasco Azevedo, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil. C. diphtheriae reference strains from the American Type Culture Collection (ATCC), Rockville, MD, USA, and from Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA, were also used in experiments: non-toxigenic C. diphtheriae subsp. mitis ATCC 27010, toxigenic C. diphtheriae subsp. mitis ATCC 27012, CDC E8392 and Park Williams 8, C. diphtheriae subsp. intermedius CDC D7920, C. diphtheriae subsp. gravis CDC E6651. Micro-organisms were identified by conventional biochemical methods (Mattos-Guaraldi & Formiga, 1998; Funke & Bernard, 2003), DNAse test (Pimenta et al., 2008b) and by the API Coryne system (bioMérieux) in accordance with the manufacturer’s instructions.

Cytotoxicity of C. diphtheriae strains, including reference strains, was also evaluated by PCR using a primer pair targeted to a portion of fragment A of the tox gene (PCR-DTA, 258 bp), as well as by the ‘gold-standard’ Vero cells cytotoxicity assay (Efstatiou et al., 1998). The multiplex PCR assay was performed using three primer pairs corresponding to dtxR gene (DtxR1F and DtxR1R, 258 bp) and fragments of portions A (Diphyt 2F and Diph 4R, 719 bp) and B (Diphyt 6F and Diph 7R, 534 bp) of the tox gene (Nakao et al., 1996). Bacterial DNA was extracted by boiling a suspension composed of a loopful of freshly cultured bacteria in 500 μl sterile water for 10 min. The PCR was performed in a 25 μl volume containing: 1 × Taq polymerase buffer, 1.5 mM MgCl₂, 200 μM dNTPs, 4 μM each of the primers, 1.25 units Taq DNA polymerase (all reagents from Gibco-BRL) and 2 μl template DNA. Thermo cycling was performed using 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min. The reaction was concluded with a final extension step of 72 °C for 10 min.

Results of the multiplex PCR of C. diphtheriae strains are shown in Fig. 1. Despite differences in the sites of isolation, biotypes and subspecies, all 84 C. diphtheriae clinical and 6 control strains tested exhibited at least one amplification product with the expected size of 258 bp, corresponding to the dtxR gene. A recent investigation indicated the use of the dtxR gene as a species-specific marker for C. diphtheriae (Pimenta et al., 2008a). In that work, results of the PCR-dtxR were 100 % positive for non-toxigenic and toxigenic C. diphtheriae strains. Conversely, the PCR-dtxR did not show an amplification signal from clinical isolates identified as non-diphtherial Corynebacterium species. In the present study, all three C. ulcerans and five C. pseudotuberculosis strains tested were negative for PCR-dtxR. PCR-dtxR completely correlated with the standard biochemical and commercial identification for all C. diphtheriae strains tested, indicating the feasibility of this reaction. In the present study, a complete agreement between results of multiplex PCR and PCR-dtxR was observed for all C. diphtheriae strains tested.

In addition to the dtxR gene amplicon, two amplification products of 719 and 534 bp corresponding to fragments of portions A and B of the tox gene, respectively, were obtained by multiplex PCR for all toxigenic strains tested. Results were 100 % concordant with those observed in PCR assays using the primers targeted to domains A or B in separate reactions (Pimenta et al., 2008a). A total of 33 clinical and 5 control strains were...
identified as toxigenic, and 51 clinical strains and ATCC 27010 as non-toxigenic by multiplex PCR. Complete agreement between the results of multiplex PCR, PCR-DTA and Vero cell cytotoxicity assays was observed for all C. diphtheriae strains tested. Toxin-gene-bearing strains (Cianciotto & Groman, 1997) were not observed among the C. diphtheriae strains tested. All non-toxigenic C. ulcerans and non-toxigenic C. pseudotuberculosis clinical strains were negative for multiplex PCR and PCR-DTA. Since C. ulcerans and C. pseudotuberculosis are not commonly related to human and animal infections in Rio de Janeiro, Brazil, additional study remains necessary in order to investigate the use of multiplex PCR for C. ulcerans and C. pseudotuberculosis strains.

The identification of C. diphtheriae should be initiated without delay following the isolation of any suspicious colonies. Phenotypic identification procedures tend to be technically demanding, or lacking in sensitivity, or else have limited evaluations. In general, these methods require at least 2 days from the selection of a suspicious organism. Important advantages of multiplex PCR over conventional biochemical procedures are its rapidity, ease of performance, the large number of strains that can be simultaneously tested for and the fact that interpretation of a PCR multiplex assay is simple. Identification by PCR proved to be reliable, and could be extremely important because it allows rapid application of control procedures and treatment of diphtheria or C. diphtheriae systemic infections. The multiplex PCR system may be incorporated into routine analyses in clinical diagnosis laboratories and/or epidemiological research.

Acknowledgements

This work was supported by CNPq, CAPES, FAPERJ, SR-2/UFBA and the Programa de Núcleo de Excelência (PRONEX) of the Brazilian Ministry of Science and Technology.

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